Original Paper Plant Biotechnology, **19** (1), 1-6 (2002)

Cloning of the S_8-*RNase* (S_8-allele) of Japanese Pear (*Pyrus pyrifolia* Nakai)

Carlos CASTILLO[1,5], Takeshi TAKASAKI[2]*, Toshihiro SAITO[3], Shigemi NORIOKA[4] and Tetsu NAKANISHI[1,2]

[1] *Graduate School of Science and Technology, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe, Hyogo 657-8501, Japan*
[2] *Faculty of Agriculture, Kobe University, 1-1 Rokkodai, Nada-ku Kobe, Hyogo 657-8501, Japan*
[3] *National Institute of Fruit Tree Science, 2-1 Fujimoto, Tsukuba, Ibaraki 305-8605, Japan*
[4] *Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan*
[5] *National Institute of Agrarian Research, Av. La Universidad s/n, Lima 12, Peru*
**Corresponding author* E-mail address: taka@kobe-u.ac.jp

Received 24 May 2001; accepted 11 September 2001

Abstract

The expression of S_8-*RNase* was confirmed in pistils of two Japanese pear cultivars, 'Ichiharawase' (S_1S_8) and 'Heiwa' (S_4S_8). The complete sequence of the S_8-*RNase* gene was determined connecting the nucleotide sequences of partial cDNA and 5' terminal genomic DNA fragments amplified by RT-PCR and genomic PCR. The S_8-*RNase* has an open reading frame of 684 nucleotides encoding 228 amino acid residues. A hypervariable region (HV) of S_8-RNase, which is quite different from those of S_1- to S_7-RNases, includes an intron of 234 bp. The similarity of deduced amino acid sequences between S_8-RNase and the seven S-RNases of Japanese pear ranged from 56.7% (S_3-RNase) to 70.2% (S_7-RNase). Based on its nucleotide sequence, we selected *Nru*I as S_8-*RNase* specific restriction endonuclease and established the PCR-RFLP system for discriminating S_1- to S_8-alleles.

Keywords: PCR-RFLP, *Pyrus pyrifolia*, Self-incompatibility, S-RNases.

Abbreviations

PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; PCR-RFLP, PCR-restriction fragment length polymorphism.

Introduction

Self-incompatibility (SI) is a widespread genetic mechanism to prevent inbreeding in plants (de Nettancourt, 1977). Japanese pear "Nashi" (*Pyrus pyrifolia* Nakai) exhibits gametophytic self-incompatibility (GSI) that is controlled by a single gene (S-locus) with multi-allelles (Kikuchi, 1929). Seven alleles (S_1 to S_7) were identified in 22 cultivars by pollination experiments (Terami *et al.*, 1946). Since then, these S-genotype assignments have been used as cross indicators for identifying the genotype of other cultivars. Several pollination studies have been performed, and S-genotypes of around 40 cultivars have been described to date (Terami *et al.*, 1946; Ogaki, 1958; Machida *et al.*, 1982; Hiratsuka *et al.*, 1998).

In Japanese pear, S-allele-associated stylar glycoproteins with RNase activity (termed S-RNases) have been identified by protein analysis (Sassa *et al.*, 1992; Ishimizu *et al.*, 1996a). S-RNase has been proved to be responsible for GSI in Japanese pear (Sassa *et al.*, 1997). The cDNAs encoding the S_1 to S_7-RNases have been isolated and sequenced from four cultivars, 'Imamuraaki' (S_1S_6), 'Nijisseiki' (S_2S_4), 'Hosui' (S_3S_5) and 'Okusankichi' (S_5S_7) (Norioka *et al.*, 1996; Ishimizu *et al.*, 1998). The diversity of their deduced amino acid sequences has allowed us to appoint the hypervariable (HV) region, which is thought to be responsible for recognition of S-alleles (Ishimizu *et al.*, 1998). The introns inserted in the HV regions have also been sequenced from genomic DNAs encoding the S_1 to S_7-RNases. Based on the nucleotide sequences within their HV region and intron, we previously proposed a PCR-RFLP system for identifying the seven S-alleles of Japanese pear (Ishimizu *et al.*, 1999).

Recently, using the PCR-RFLP system, we reconsidered the S-genotype assignments of six Japa-

nese pear cultivars, 'Akaho', 'Tanzawa', 'Kimizukawase', 'Choju', 'Ichiharawase' and 'Meigetsu' (Castillo *et al.*, 2001). 'Ichiharawase' and 'Meigetsu' were both identified to have the *S*-genotype of S_1S_5 by pollination tests (Terami *et al.*, 1946). From 'Ichiharawase' and 'Meigetsu', the *S_1-RNase* fragment (367 bp) and a new S-RNase fragment (436 bp) were amplified by PCR-RFLP analysis, but the S_5-RNase was not. The new *S-RNase* fragment presented a unique size and digestion pattern compared to the S_1- to S_7-*RNases*, and its partial deduced amino acid sequence included a quite different HV region. In addition, the intron inserted within the HV region had a different size. These distinctions led us to designate the new *S-RNase* fragment as the *S_8-RNase*, and as a result, the *S*-genotype of S_1S_8 was reassigned to 'Ichiharawase' and 'Meigetsu' (Castillo *et al.*, 2001).

In this study, we confirmed the expression of *S_8-RNase* in pistils of two Japanese pear cultivars by RT-PCR analysis and determined the complete nucleotide sequence of *S_8-RNase* including the intron. Based on its nucleotide sequence, we selected an *S_8-RNase* specific restriction endonuclease and established the PCR-RFLP system for discriminating among S_1- to S_8-alleles.

Material and Methods

Plant Material and DNA

Young leaves of seven Japanese pear cultivars, 'Ichiharawase', 'Meigetsu', 'Heiwa', 'Imamuraaki','Nijisseiki', 'Hosui' and 'Okusankichi' were collected in spring at the National Institute of Fruit Tree Science, Ministry of Agriculture, Forestry and Fisheries of Japan in Tsukuba. The leaves were stored at −80 ℃. Genomic DNA was extracted from 0.1-0.2 g of leaves according to the method of Doyle and Doyle (1987). Flowers of 'Ichiharawase' and 'Heiwa' were collected at the white bud stage. Pistils were removed, frozen in liquid nitrogen and then stored at − 80 ℃ until use.

Isolation of mRNA

Total RNA was extracted from pistils of 'Ichiharawase' (S_1S_8) and 'Heiwa' (S_4S_8) according to the method described by Chomczynski and Sacchi (1987). Two hundred pistils were ground in liquid nitrogen and 5 ml of the extract buffer (5 M guanidine isothiocyanate, 10 mM Tris-HCl pH 7.5, 10 mM EDTA, 30 mg ml^{-1} Polyclar AT, 5% 2-mercaptoethanol and 0.5% sodium N-sauroyl sarsosinate) was added. After centrifugation, the supernatant was recovered and mixed well with 0.2 ml of 2 M NaOAc (pH 4.0), 2 ml of water-saturated phenol, and 1 ml of chloroform-isoamyl alcohol (24:1). The mixture was cooled on ice for 15 min. After centrifugation, the aqueous phase was mixed with 4.5 ml of isopropanol and held at −20 ℃ for 2 h to precipitate RNA. The RNA pellet was washed with 75% ethanol, dried for 1 h at room temperature, and then it was dissolved in 0.8 ml of distilled water. mRNAs were isolated from total RNA using the Micro-FastTrack™ 2.0 mRNA Isolation Kit (Invitrogen) according to the manufacturer's instructions.

RT-PCR

RT-PCR was carried out using the Titan™ One Tube RT-PCR system (Roche Diagnostics) according to the manufacturer's instructions. mRNA was reverse-transcribed for 30 min at 50 ℃ to synthesize first-strand cDNA. PCR amplification was performed with a set of primers, 'FTQQYQ' (5'-TTTACGCAGCAATATCAG-3') and adapter primer *Not*I-$(dT)_{18}$ (Amersham-Pharmacia) for 10 cycles of denaturation for 30 s at 94 ℃ , annealing for 30 s at 55 ℃ and extension for 45 s at 68 ℃, following 25 cycles of denaturation for 30 s at 94 ℃, annealing for 30 s at 55 ℃ and initial extension for 45 s at 68 ℃, adding 5 s for each cycle. A final extension step for 7 min at 68 ℃ was performed. Nested PCR was carried out with a set of primers, 'CNSNPT' (5'-TGCAACTCYAAWCGTACTC-'3) and *Not*I-dT (5'-AACTGGAAGAATTCGCGGC-CGCAGGAT T-'3) for 10 cycles of denaturation for 15 s at 94 ℃, annealing for 30 s at 60 ℃ and extension for 1 min at 70 ℃ . Followed by 20 cycles of denaturation for 15 s at 94 ℃, annealing for 30 s at 60 ℃ and extension for 1.5 min at 70 ℃. Then, a final extension step for 7 min at 70 ℃. After PCR-products were digested with *Eco*RI (which cleavages S_1- and *S_4-RNases* specifically), the undigested *S_8-RNase* fragment was directly sequenced.

Amplification of S-RNase fragment from genomic DNA

S-RNase fragments were amplified from genomic DNA by PCR with *S-RNase* specific primers, '5'-32 bp' (5'-TGCCTCGCTCTTGAACAAA-3'), 'FTQQYQ' and 'anti-IIWPNV' (5'-AC(A/G)TT-CGGCCAAATAATT-3'). Genomic DNA (50 ng) was mixed with 0.3 μM of each primer, 200 μM dNTP, 1x PCR-buffer, 1U Taq polymerase and distilled water up to final volume of 30 μl. PCR amplification was carried out for 10 cycles of denaturation for 15 s at 94 ℃ , annealing for 30 s at 48 ℃ and extension for 1 min at 70 ℃ , following 20 cycles of denaturation for 15 s at 94 ℃, annealing for 30 s at 48 ℃ and extension for 1.5 min at 70 ℃,

with a final extension for 7 min at 70 ℃.

Sequencing analysis

PCR products were run on 2% agarose gels and each fragment was isolated using the GENECLEAN II Kit (Bio 101, Inc.). Nucleotide sequences were determined by the dideoxy-nucleotide chain termination method, with the primers described above, using an ABI PRISM™ 310 DNA capillary sequencer. All data were analyzed with DNASIS-Mac software (Hitachi Software Engineering Co.).

Restriction endonuclease digestion

S-allele specific restriction endonuclease digestion was carried out under conditions described previously (Castillo *et al.*, 2001). *S-RNase* fragments were amplified from the seven allele set cultivars, 'Imamuraaki' (S_1S_6), 'Nijisseiki' (S_2S_4), 'Hosui' (S_3S_5) and 'Okusankichi' (S_5S_7), as well as from 'Ichiharawase' (S_1S_8), 'Meigetsu' (S_1S_8) and 'Heiwa' (S_4S_8) harboring S_8-allele. The amplified *S-RNase* fragments were digested with *S*-allele specific restriction endonucleases (*Sfc*I, *Ppu*MI, *Nde*, I *Alw*NI, *Hinc*II, *Acc*II and *Nru*I) and digested fragments were electrophoresed on 2% agarose gels.

Results and Discussion

To investigate the expression of S_8-*RNase* in pistils of 'Ichiharawase' and 'Heiwa', we amplified cDNA corresponding to S_8-*RNase* by RT-PCR from mRNA of both cultivars. Two fragments of almost the same size (around 750 bp) were amplified with primers 'FTQQYQ' and *Not*I-$(dT)_{18}$ (**Fig. 1A**). RT-PCR products were then used as the template for nested PCR amplification with 'CNSNPT' and *Not*I-dT primers. The nested PCR yielded two *S-RNase* fragments of about 720 bp that corresponded with the expected sizes of partial *S-RNase* cDNAs. Because the amplicons from 'Ichiharawase' and 'Heiwa' also included the S_1- and S_4-*RNase* cDNA fragments, respectively, they were digested with *Eco*RI whose recognition site is present within the HV region of S_1- and S_4-*RNases* but not within that of S_8-*RNase*. The digested *S-RNase* fragments were electrophoresed on 2% agarose gel, and then undigested S_8-*RNase* fragments were extracted from the gel and sequenced. The partial S_8-*RNase* cDNAs (558 bp) amplified from 'Ichiharawase' and 'Heiwa' were found to have the same nucleotide sequence. This indicates that S_8-*RNase* is expressed in pistils of 'Ichiharawase' and 'Heiwa'.

To obtain a complete nucleotide sequence of the S_8-*RNase* gene, we amplified the 5' terminal region of S_8-*RNase* from genomic DNA of 'Ichiharawase', 'Meigetsu' and 'Heiwa' by PCR with a set of primers, '5'-32bp' and 'anti-IIWPNV'. The '5'-32bp' primer was designed based on conserved nucleotide sequences between the initiation codon (ATG) and the putative TATA box in the 5' flanking region of Japanese pear S_2-, S_3-, S_4- and S_5-*RNase* genes (Ushijima *et al.*, 1998; Norioka *et al.*, 2001). Only one fragment of 565 bp was amplified from 'Ichiharawase' and 'Meigetsu', while two fragments of 565 bp and 496 bp were amplified from 'Heiwa' (**Fig. 1B**). The 565 bp fragment corresponds to the 5' terminal region of S_8-*RNase* because the intron of S_8-*RNase* is larger than that of S_4-*RNase* (Castillo *et al.*, 2001). The failure to amplify the S_1-*RNase* from 'Ichiharawase' and 'Meigetsu' may be due to mismatches between the '5'-32bp' primer and the 5' flanking region of S_1-*RNase*. The 5' terminal fragments of S_8-*RNase* were extracted from the gel and sequenced. The 5' region of each fragment, except the upstream region of the initiation codon, was found to have the same 533 bp nucleotide sequence including the intron.

The complete nucleotide sequence of the S_8-*RNase* was established by overlapping sequences between the partial cDNA fragment of 558 bp and 5' terminal genomic DNA fragment of 533 bp. S_8-*RNase* contains an open reading frame of 684 bp

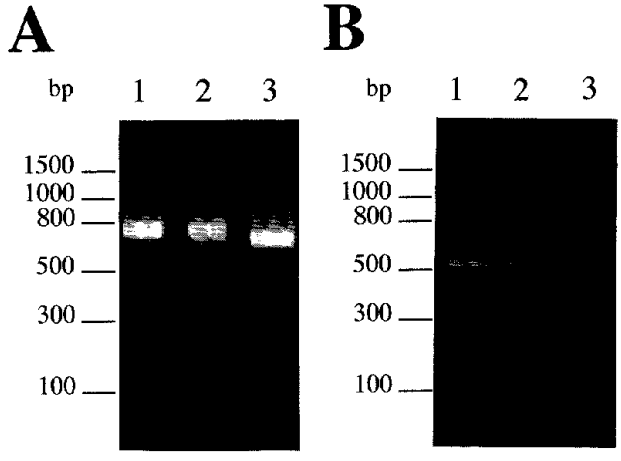

Fig. 1 *S-RNase* fragments amplified from mRNA and genomic DNA of Japanese pear cultivars. (A) The cDNA fragments of *S-RNase* amplified from 'Ichiharawase' by RT-PCR with 'FTQQYQ' and *Not*I-$(dT)_{18}$ primers (lane 1) and by nested PCR with 'CNSNPT' and *Not*I-dT primers (lane 2), and digested with *Eco*RI (lane 3). (B) The 5' teminal *S-RNase* fragments amplified from genomic DNA of 'Ichiharawase' (lane 1), 'Meigetsu' (lane 2) and 'Heiwa' (lane 3) by PCR with '5'-32bp' and 'anti-IIWPNV' primers. *S-RNase* fragments were electrophoresed on 2% agarose gels.

encoding 228 amino acid residues. There is a single intron of 234 bp in S_8-*RNase* located between amino acids 85 and 86 (**Fig. 2**). S_8-RNase shows one HV region and five conserved regions (C1 through C5) described for seven *S*-RNase of Japanese pear (Norioka *et al.*, 1996; Ishimizu *et al.*, 1998). The S_8-RNase shares 98 perfectly conserved amino acid residues with Japanese pear *S*-RNases.

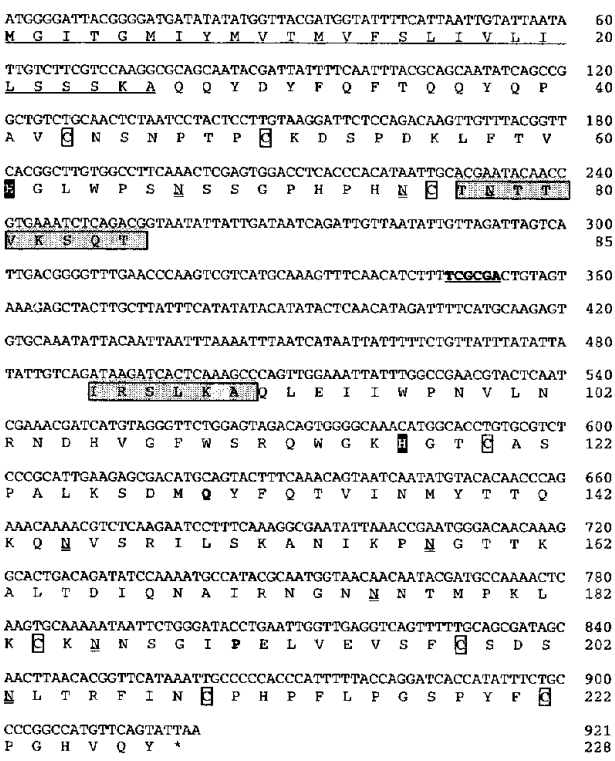

Fig. 2 Nucleotide and deduced amino acid sequences of S_8-*RNase* gene of Japanese pear. The putative signal peptide is underlined. Conserved cysteine residues are boxed. Histidine residues essential for the RNase activity are boxed in black. The HV region is boxed and shadowed. Potential N-glycosilation sites are double underlined. Punctual amino acid substitutions are in bold face. The *Nru*I site is indicated in underlined bold face. Asterisk indicates stop codon.

The S_8-RNase shows a high homology, ranging from 56.7% (S_3-RNase) to 70.2% (S_7-RNase). A putative signal peptide of S_8-RNase is predicted by Neural Networks (Nielsen *et al.*, 1997) and its most likely cleavage site is indicated between amino acids 26 and 27. The predicted mature S_8-RNase protein has a calculated pI value of 9.19 (Skoog and Wichman, 1986) that agrees with the basic nature of S_1- to S_7-RNases of Japanese pear (Sassa *et al.*, 1993; Ishimizu *et al.*, 1996a). S_8-RNase possessed the primary structural features of *S*-RNases of Japanese pear, two histidine residues (His-61 and His-117) essential for T2/S type RNase activity (Kawata *et al.*, 1989) and eight cysteine residues forming four disulfide bridges important for the formation or stabilization of their tertiary structure (Ishimizu *et al.*, 1996b). The S_8-RNase also possessed eight potential N-glycosilation sites with the consensus sequence Asn-X-Ser/Thr (X is not Pro and Asp), including Asn-145 the only conserved in rosaceaous *S*-RNases and whose glycans may be important for the folding of the core structure (Ishimizu *et al.*, 1998). The S_8-RNase shows only 31.9% of homology with a non-*S*-RNase isolated from the pistils of 'Nijisseiki' by Norioka *et al.* (1996). All the features of S_8-RNase, as well as the primer specificity, the cultivar specificity and its Mendelian inheritance described previously (Castillo *et al.*, 2001), discard the possibility of S_8-RNase being a non-*S*-RNase protein.

Histidine (H) and Threonine (T), which are completely conserved among S_1- to S_7-RNases in Japanese pear were substituted for Gln-130 (Q) and

Table 1 Analysis of S_1- to S_8-RNases of Japanese pear by PCR-RFLP.

Cultivar	S_2	*Sfc*I S_1, S_8	*Ppu*MI S_3, S_5	*Nde*I S_4	*Alw*NI S_5	*Hinc*II S_6	*Acc*II S_6, S_7, S_8	*Nru*I S_8	*S*-genotype PCR-RFLP
Imamuraaki	-	+	-	-	-	+	+	-	S_1S_6
Nijisseiki	o	-	-	+	-	-	-	-	S_2S_4
Hosui	-	-	++	-	+	-	-	-	S_3S_5
Okusankichi	-	-	+	-	+	-	+	-	S_5S_7
Ichiharawase	-	++	-	-	-	-	+	+	S_1S_8
Meigetsu	-	++	-	-	-	-	+	+	S_1S_8
Heiwa	-	+	-	+	-	-	+	+	S_4S_8

O:S_2-*RNase* fragment amplified by PCR

++:Two *S*-*RNase* fragments were digested with restriction endonucleases

+:One of two *S*-*RNase* fragments was digested with restriction endonucleases

-:*S*-*RNase* fragments were not digested with restriction endonucleases

Pro-191 (P), respectively, in the deduced amino acid sequence of S_8-RNase. The HV region is considered to control the allelic specificity in self-incompatible reactions (Matton *et al.*, 1997). The HV region of S_8-RNase is between the 77th and 91st residues and is quite different from those of S_1 to S_7-RNases. In this region, the S_8-RNase has the highest number of substitutions (14 amino acids) with S_3- and S_5-RNases and the lowest (7 amino acids) with S_6-RNase.

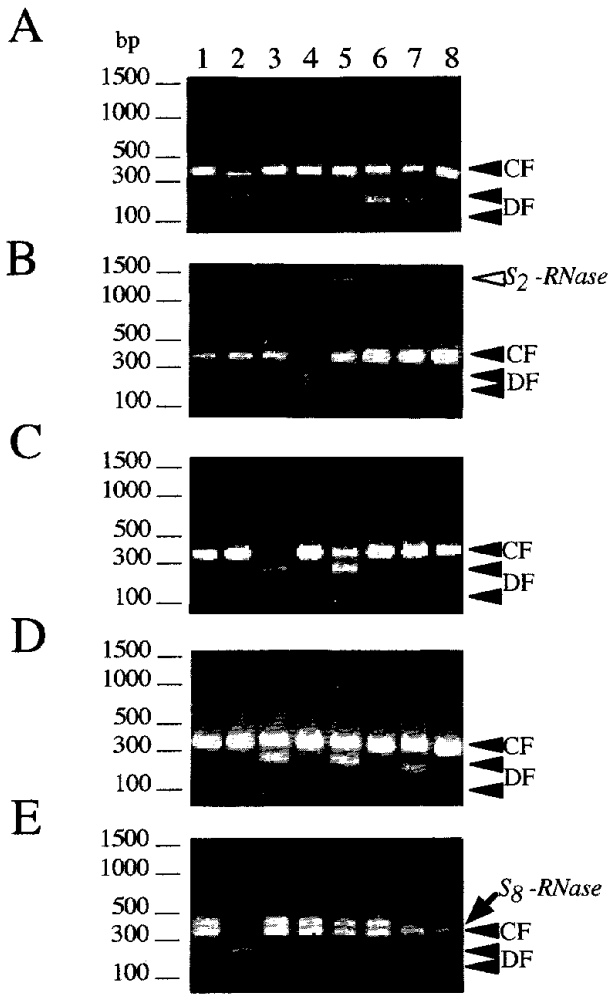

Fig. 3 Analyses of the digestion pattern of *S-RNase* fragments in Japanese pear cultivars haboring S_1- to S_8-allele. (A) 'Imamuraaki' (S_1S_6), (B) 'Nijisseiki' (S_2S_4), (C) 'Hosui' (S_3S_5), (D) 'Okusankichi' (S_5S_7), (E) 'Imamuraaki' (S_1S_8). The lanes in each gel show, from left to right, 1: amplified *S-RNase* fragments, 2: *Sfc*I (S_1, S_8-specific), 3: *Ppu*MI (S_3, S_5-specific), 4: *Nde*I (S_4-specific), 5: *Alw*NI (S_5-specific), 6: *Hin*cII (S_6-specific), 7: AccII (S_6, S_7, S_8-specific) and 8: *Nru*I (S_8-specific). CF: common fragments, DF: digested fragments. The S_2-*RNase* fragment is shown by a white arrowhead. The S_8-*RNase* fragment is shown by an arrow. Each digested fragment was electrophoresed on 2% agarose gels.

Cultivars with S_8-allele are compatible with all tester cultivars harboring any combination of the seven other alleles, thus it is difficult that *S*-genotype assignment of these cultivars be identified by pollination tests. The PCR-RFLP system has proved to be a reliable method for *S*-genotype typing (Ishimizu et al, 1999; Castillo *et al.*, 2001). The PCR-RFLP system with 'FTQQYQ' and 'anti-IIWPNV' primers amplified the S_8-*RNase* fragment, which was slightly different in size from S_1- to S_7-*RNase* fragments and was cleavaged by *Sfc*I and *Acc*II specific for S_1 and S_6, S_7-*RNase*, respectively, producing a distinct but complicated digestion pattern (Castillo *et al.*, 2001). To obtain a clear discrimination of the S_8-*RNase*, we selected a new restriction endonuclease, *Nru*I that digests the S_8-*RNase* fragment producing 251 bp and 185 bp fragments. The PCR-RFLP system with the addition of *Nru*I, was tested by determining *S*-genotypes of the seven allele set cultivars, 'Imamuraaki' (S_1S_6), 'Nijisseiki' (S_2S_4), 'Hosui' (S_3S_5), 'Okusankichi' (S_5S_7) and three cultivars, 'Ichiharawase' (S_1S_8), 'Meigetsu' (S_1S_8) and 'Heiwa' (S_4S_8) (**Table 1**). *Nru*I digested only the S_8-*RNase* fragment but not the S_1- to S_7-RNase fragments (**Fig. 3**).

In this study, we confirmed the expression of S_8-*RNase* in pistils of 'Ichiharawase' and 'Heiwa', and determined the complete nucleotide sequence of the S_8-*RNase* including its intron. Based on the nucleotide sequence, we selected an S_8-*RNase* specific restriction endonuclease, *Nru*I, and established an PCR-RFLP system for identifying *S*-genotypes of Japanese pear cultivars harboring S_1 to S_8 alleles.

Acknowledgement

This work was supported in part by Grants-in-Aid for Special Research on Priority Areas (10660025 and 13760005) from the Ministry of Education, Science, Culture and Sports, Japan.

References

Castillo, C., Takasaki, T., Saito, T., Yoshimura, Y., Norioka, S., Nakanishi, T., 2001. Reconsideration of *S*-genotype assignments, and discovery of a new allele based on *S-RNase* PCR-RFLPs in Japanese pear cultivars. Breed. Sci., **51**: 5-11.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., **162**: 156-159.

Doyle, J. J., Doyle, J. L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull., **19**: 11-15.

Hiratsuka, S., Kubo, T., Okada, Y., 1998. Estimation of self-incompatibility genotype in Japanese pear cultivars by stylar protein analysis. J. Japan. Soc. Hort. Sci., **67**: 491

-496.

Ishimizu, T., Sato, Y., Saito, T., Yoshimura, Y., Norioka, S., Nakanishi, T., Sakiyama, F., 1996a. Identification and partial amino acid sequences of seven S-RNases associated with self-incompatibility of Japanese pear, *Pyrus pyrifolia* Nakai. J. Biochem., **120**: 326-334.

Ishimizu, T., Norioka, S., Kanai, M., Clarke, A. E., Sakiyama, F., 1996b. Location of cysteine and cystine residues in S-ribonucleases associated with gametophytic self-incompatibility. Eur. J. Biochem., **242**: 627-635.

Ishimizu, T., Shinkawa, T., Sakiyama, F., Norioka, S., 1998. Primary structural features of rosaceous *S*-RNases associated with gametophytic self-incompatibility. Plant Mol. Biol., **37**: 931-941.

Ishimizu, T., Inoue, K., Shimonaka, M., Saito, T., Terai, O., Norioka, S., 1999. PCR-based method for identifying the *S*-genotypes of Japanese pear cultivars. Theor. Appl. Genet., **98**: 961-967.

Kawata, Y., Sakiyama, F., Hayashi, F., Kyogoku, Y., 1989. Identification of two essential histidine residues of ribonuclease T_2 from *Aspergillus oryzae*. Eur. J. Biochem., **187**: 244-262.

Kikuchi, A., 1929. Investigations in 1927 and 1928. 1. Paterclinical incompatibility in the Japanese pear. J. Okitsu Hort. Soc., **24**: 1-6 (in Japanese).

Machida, Y., Sato, Y., Kozaki, I., Seiki, K., 1982. *S*-genotypes of several cultivars of Japanese pear and the question of the parents of 'Hosui'. Abstr. Japan. Soc. Hort. Sci. Autumm Meet., 58-59 (in Japanese).

Matton, D., Maes, O., Laublin, G., Xike, Q., Bertrand, C., Morse, D., Cappadocia, M., 1997. Hypervariable domains of self-incompatibility RNases mediate allele-specific pollen recognition. Plant Cell, **9**: 1757-1766.

de Nettancourt, D., 1977. Incompatibility in Angiosperm. Springer-Verlag. Berlin.

Nielsen, H., Engelbrecht, J., Brunak, S., von Heijne, G., 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng., **10**:1-6.

Norioka, N., Norioka, S., Ohnishi, Y., Ishimizu, T., Oneyama, C., Nakanishi, T., Sakiyama, F., 1996. Molecular cloning and nucleotide sequences of cDNAs encoding S-allele specific stylar RNases in a self-incompatible cultivar and its self-compatible mutant of Japanese pear, *Pyrus pyrifolia* Nakai. J. Biochem., **120**: 335-345.

Norioka, N., Katayama, H., Matsuki, T., Ishimizu, T., Takasaki, T., Nakanishi, T., Norioka, S., 2001. Sequence comparison of the 5' flanking regions of Japanese pear (*Pyrus pyrifolia*) S-RNases associated with gametophytic self-incompatibility. Sex. Plant Reprod., **13**: 289-291.

Ogaki, C., 1958. The sterility-factors in new varieties of the Japanese pear (*Pyrus serotina* Rehd. Var. culta Rehd.). Res. Rep. Kanagawa Agr. Sta. Hort. Inst., **5**: 23-26 (in Japanese with English summary).

Sassa, H., Hirano, H., Ikehashi, H., 1992. Self-incompatibility-related RNases in styles of Japanese pear (*Pyrus serotina* Rehd.). Plant Cell Physiol., **33**: 811-814.

Sassa, H., Hirano, H., Ikehashi, H., 1993. Identification and characterization of stylar glycoproteins associated with self-incompatibility genes of Japanese pear, *Pyrus serotina* Rehd. Mol. Gen. Genet., **241**: 17-25.

Sassa, H., Hirano, H., Nisho, T., Koba, T., 1997. Style-specific self-compatible mutation caused by deletion of the *S-RNase* gene in Japanese pear (*Pyrus serotina*). Plant J., **12**: 223-227.

Skoog, B., Wichman, A., 1986. Calculation of the isoelectric points of polypeptides from the amino acid composition. Trends Anal. Chem., **5**: 82-83.

Terami, H., Torikata, H., Shimazu, Y., 1946. Analysis of the sterility factors existing in varieties of the Japanes pear (*Pyrus serotina* Rehd. *var. culta* Rehd). Studies Hort. Inst. Kyoto Imp. Univ., **3**: 267-271 (in Japanese with English summary).

Ushijima, K., Sassa, H., Hirano, H., 1998. Characterization of the flanking regions of the *S-RNase* genes of Japanese pear (*Pyrus serotina*) and apple (*Malus x domestica*). Gene, **211**: 159-167.